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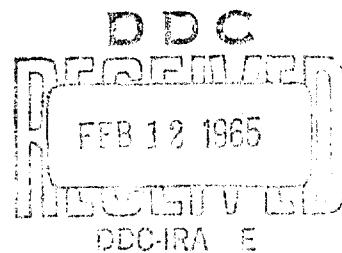
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TECHNICAL MANUSCRIPT 171

PURIFICATION AND CHARACTERIZATION OF POISON PRODUCED BY GONYAULAX CATENELLA IN AXENIC CULTURE

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PURIFICATION AND CHARACTERIZATION OF POISON
PRODUCED BY GONYAULAX CATENELLA
IN AXENIC CULTURE

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ABSTRACT

The paralytic poison produced by the dinoflagellate Gonyaulax catenella grown in axenic culture has been isolated in pure form. A study of its chemical, physical, and biological properties indicates that it is very similar if not identical to the poison isolated from toxic Alaska butter clams and California sea mussels.

I. INTRODUCTION

The paralytic poison sometimes occurring in California mussels was found by Sommer et al.¹ to result from the mussels' feeding on a particular dinoflagellate, Gonyaulax catenella. Those workers collected the dinoflagellates and discovered that they contained a poison similar in toxic properties to that occurring in the mussels. When mussels or other shellfish containing this poison are consumed by humans a rapid onset of paralysis results that often terminates in death. The occurrence of this poison in shellfish presents a public health problem and its significance and medical implications are described by Sommer and Meyer² (1937), Meyer³, Huttner and McLaughlin⁴ and McFarren et al.⁵ In recent years the poison was isolated in pure form from mussels and also from Alaska butter clams. Riegel et al.⁶ examined concentrates of the poison from G. catenella collected from the Pacific Ocean and found many properties of the poison in these extracts to be similar to those of the poison from mussels. Burke et al.⁸ have studied the chromatographic behavior of crude extracts of cultured G. catenella cells and found the poison to chromatograph similarly to mussel poison.

Besides the association of the poison in the California mussels with G. catenella, the poison occurring in the scallops in the Bay of Fundy has been associated with the occurrence of G. tamerensis^{9,10} and the poison in the Belgian mussels in the North Sea with the occurrence of Pyrodinium phoneus.¹¹ The origin of the poison in the Alaska butter clam is not understood but some observations indicate that G. catenella may be involved.¹²

This paper describes the isolation, purification, and partial characterization of the poison from G. catenella cells obtained from axenic culture and presents data indicating that this poison is very similar if not identical in chemical structure to the poison from mussels and clams.

II. MATERIALS AND METHODS

A pure culture of Gonyaulax catenella (free of bacteria) was obtained from Dr. L. Provasoli, Haskins Laboratories, New York, N.Y.* The culture medium was similar to that described by Provasoli and used by Burke et al.⁸ Cultures were initiated by inoculating 10 ml of medium with one ml of the stock culture containing about 20,000 organisms per ml and incubating at 13 C for 12 days. This culture was used as the inoculum for 100 ml of the culture medium, which in turn was used as the inoculum for one liter of medium. This culture was incubated for 17 days at 13 C when the cell count per ml was about 30,000. At that time the cultured cells were collected by filtration on a fast-flowing filter paper (Eaton-Dikeman 615), ground in a Waring Blender with sufficient dilute HCl to make a thick slurry (pH 2 to 3), and filtered with suction. This solution was usually cloudy and was clarified by mixing with Celite 545 and filtering. The clear yellow-green filtrate constituted a crude extract of the poison and usually contained between 20 and 100 mouse units (MU) of poison per ml.

Toxicities of all preparations were determined by injecting serial dilutions of the poison solution intraperitoneally into white mice** weighing between 18 and 22 grams and measuring the time to death to determine the potency.¹³ The results were expressed as MU per ml or with the total solids per ml (specific toxicity) as MU per mg of solids. Basically the mouse unit is defined as the amount that will kill a 20-gram mouse in 15 minutes.² G. catenella cell counts in the cultures were made with a Howard mold count slide. Infrared measurements were made in KBr pellets, using a Perkin-Elmer Model 12 C spectrophotometer. Absorbance in the ultraviolet was measured in 0.001 N HCl using a Model DU Beckman spectrophotometer. Titrations were carried out with a Radiometer TTTIC equipped with a PHA630T scale expander, using 3 ml of a solution of the poison at a concentration of 9 mg per ml in 0.001 N HCl (free of carbon dioxide) and with 0.25 N sodium hydroxide under an atmosphere of nitrogen. Optical rotation measurements were made in 0.001 N HCl in a 20-cm tube. Reduction with hydrogen was carried out in a Warburg respirometer at one atmosphere of hydrogen pressure using platinum black adsorbed on charcoal.¹⁴ Diffusion coefficients were measured in the Northrop diffusion cell¹⁵ by observing the rate of diffusion of the biological activity through the sintered glass disc of that apparatus. Color tests were carried out as described by Albanese and Frankston¹⁶ for the Sakaguchi test, Benedict and Behre¹⁷ for the Benedict-Behre test, and Bonsness and Tausky¹⁸ for the Jaffe test.

* This organism was originally obtained in 1949 from the Pacific Ocean (Monterey Bay) by Herman Sommer and Lucile Foster of the Hooper Foundation, University of California Medical Center, San Francisco.

** In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

III. PURIFICATION AND CHARACTERIZATION

The crude extract of the poison was passed through a column of Amberlite XE-64 in the sodium form to remove the poison. About 100 grams of the resin were used per million MU of poison in the crude extract. The poison was fractionally eluted from the resin with 0.3 M acetic acid. A recovery of at least 90% was achieved with a toxicity of 400 or more MU per mg of solids. Because the properties of this poison were found similar to those of the poison in mussels, purification was attempted by a method similar to that used to purify the poison from mussels.⁶ The fractions containing the bulk of the poison in 0.3 M acetic acid were pooled, concentrated under vacuum to remove most of the acid, and adjusted to pH 4.5. The poison in the pooled fractions was adsorbed on a column of XE-64 in the acid form, followed by chromatographic elution of the poison with 0.3 M acetic acid. The fractions having a toxicity of 1000 or more MU per mg of solids were pooled and rechromatographed in the same manner. Usually the specific toxicity after passing through the column a second time was about 3000 MU. The fractions at about this potency were pooled, evaporated under vacuum to remove most of the acetic acid, and acidified with hydrochloric acid to about pH 2. The solution was again evaporated under vacuum to remove all of the acetic acid and to convert the poison from the acetate to the chloride salt. The pH should remain between 2 and 3 after all traces of acetic acid have been distilled off. The solution was then treated with small amounts of well-washed Amberlite IR-45 to bring the pH to 4.5 and the poison was lyophilized. The dried poison was dissolved in absolute ethanol and chromatographed on acid-washed alumina as described for the purification of mussel poison. The best fractions from the chromatography on alumina had a specific toxicity of 5100 and a specific rotation of 128. The over-all yield was about 50% based on the poison content of the crude extract.

Further chromatography of the poison from these fractions on the alumina did not significantly increase the specific toxicity or change the specific rotation of the poison. Chromatography of the preparation on Whatman No. 1 paper strips using solvent systems of phenol-water (4:1) and t-butyl alcohol-acetic acid-water (1:1:1) as described by Mold et al.¹⁹ showed that all substances detectable with the Jaffe reagent and by assay in mice moved as a single band with an R_f of 0.26 to 0.30. Continued studies with this poison clearly indicated that the substance isolated by the above procedure was highly purified and similar in its chromatographic behavior to purified mussel poison. Studies therefore were undertaken to compare this poison with purified clam and mussel poisons. The results of these studies are summarized in Table I. All chemical reactions and physical measurements on the three poisons

TABLE 1. COMPARISON OF PROPERTIES OF POISON FROM CULTURED
GONYAULAX CATENELLA CELLS WITH "POISON" FROM MUSSELS AND CLAMS

Property	Clam Poison	Mussel Poison	<u>G. catenella</u> Poison
Toxicity, MU/mg ^a /	5200	5300	5100
Specific optical rotation	128	130	128
pKa	8.3; 11.5	8.3; 11.5	8.2; 11.5 ^a
N content (Kjeldehl)	26.3	26.3	26.3
Sakaguchi test	negative	negative	negative
Benedict-Behre test	positive	positive	positive
Jaffe test	positive	positive	positive
Adsorption in UV and visible	none	none	none
Infrared adsorption, μ	3, 6 & 9	3, 6 & 9	3, 6 & 9
Reduction with H_2	nontoxic	nontoxic	nontoxic
Diffusion coefficient	4.9×10^{-6}	4.9×10^{-6}	4.8×10^{-6}

a. All of these bioassay values are within experimental error of the value
 5500 ± 500 MU/mg solids reported previously for clam and mussel poisons.¹³

were identical in every respect, indicating that their structures must be very similar if not identical. The infrared spectra of purified mussel poison and purified G. catenella poison are shown in Figure 1, and as far as can be determined they are identical. Reduction of the poisons with hydrogen resulted in a loss of 95 to 100% of the toxicity when one mole of the gas was consumed for each mole of poison. The infrared spectra of the reduced poison (nontoxic) showed some loss of absorption at 5.6 and 8.7 μ , which is identical to that obtained with clam and mussel poisons. The specific toxicity of the three poisons was identical within experimental error of the bioassay. The specific optical rotation is also the same for all poisons within the error of the measurements.

Elemental analyses of the purified G. catenella poison as the dihydrochloride salt showed 31.8% carbon, 5.3% hydrogen, and 26.3% nitrogen. The diffusion coefficient of the poison ($4.8 \times 10^{-8} \text{ cm}^2 \text{ sec}^{-1}$) indicates a molecular weight of 300 to 400. Titration of the poison showed two titratable groups; one with a pK_a at 8.2 and the other at about 11.5. A sample of 27 mg (hydrochloride salt) required 0.140 ml of 0.25 M NaOH to bring the pH to the first inflection point at 8.25; 0.28 ml to pH 9.60 for the inflection point between the groups, and 0.525 ml to pH 11.5 for the approximate inflection point of the second group. The molecular weight calculated from the titration value of the first group at pH 9.60 was 386. This titration showed the chlorine content to be 18.4%. If it is assumed that oxygen makes up the remainder of the elemental composition (18.2%) the molecular formula should be $C_{10}H_{17}N_7O_4 \cdot 2HCl$ with a molecular weight of 372. This value within experimental error is exactly that obtained for the clam and mussel poisons.

Recently Rapoport et al.²⁰ has proposed a structure for the clam poison (Saxatoxin). Although all evidence obtained thus far indicates that the G. catenella poison is identical to the mussel and clam poisons, absolute proof of identity can come only by studies of its elemental structure.

On the basis of these studies, the poison from the cultured G. catenella must be the same substance as that produced by this organism in the natural state in the Pacific Ocean, and when the poison is bound in the dark gland or hepatopancreas of the mussel and probably in the siphon of the clam, no apparent change in structure results.

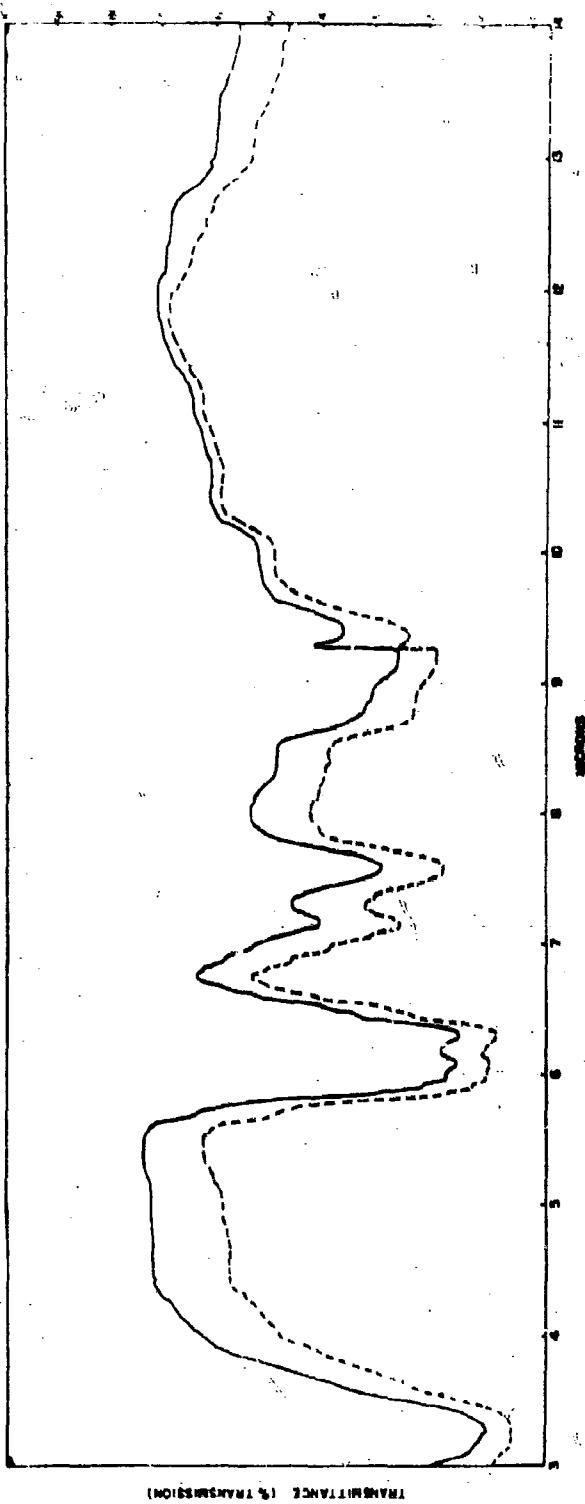


Figure 1. Infrared Spectra of Purified G. catenella Poison (Solid Line) and Purified Mussel Poison (Broken Line). The break in the curves at 9.3 μ is caused by a change from one photocell to another for the longer wave lengths.

There is some question regarding the origin of the poison in clams but the poison in California sea mussels, as pointed out previously, is believed to result from the mussels' feeding on G. catenella. The results presented in this paper definitely confirm the relationship to the mussels and add credence to the possibility that the same relationship exists with the Alaska butter clams.

The purified clam and mussel poisons are among the most potent poisons known to man. Their unique physiological action has been described by several investigators. Physiological studies on the purified poison from G. catenella should contribute valuable information on the nature of the poison in this organism and enable comparisons to be made with the poison found in several species of shellfish.

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